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The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) Polyester synthase gene and process for producing polyester

(57) The present invention relates to a polyester synthase gene coding for a polypeptide containing the amino acid sequence of SEQ ID NO:2 or a sequence where in said amino acid sequence, one or more amino acids are deleted, replaced or added, said polypeptide bringing about polyester synthase activity; a gene expression cassette comprising the polyester synthase gene and either of open reading frames located upstream and downstream of said gene; a recombinant vector comprising the gene expression cassette; a transformant transformed with the recombinant vector; and a process for producing polyester by culturing the transformant in a medium and recovering polyester from the resulting culture.

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Description

Field of the Invention

The present invention relates to a polyester synthase gene, a recombinant vector containing the gene, a transformant carrying the recombinant vector, and a process for producing polyester by use of the transformant.

Background of the Invention

It is known that a large number of microorganisms biosynthesize poly-3-hydroxybutyrate (P(3HB)) and store it in the form of ultrafine particles as an energy source in the body. P(3HB) extracted from microorganisms is a thermoplastic polymer with a melting temperature of about 180 °C, and because of its excellent biodegradability and biocompatibility it is drawing attention as "green" plastic for preservation of the environment. Further, P(3HB) is "green" plastic which can be synthesized from regenerable carbon resources including sugars and vegetable oils by various microorganisms. However, P(3HB) is a highly crystalline polymer and thus has the problem in physical properties of inferior resistance to impact, so its practical application has never been attempted.

Recently, polyester P(3HB-co-3HH) as a random copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HH) and a process for producing the same have been studied and developed, and these are described in e.g. Japanese Patent Laid Open Publication Nos. 93049/1993 and 265065/1995 respectively. In these publications, the P(3HB-co-3HH) copolymer is produced from alkanoic acids or olive oil by fermentation with Aeromonas caviae isolated from soil. It is revealed that because the degree of crystallinity of the P(3HB-co-3HH) copolymer produced through fermentation is reduced with an increasing ratio of the 3HH unit in it, so that the copolymer becomes a soft polymeric material excellent in thermostability and formability and can be manufactured into strong yarn or transparent flexible film (Y. Doi, S. Kitamura, H. Abe, Macromolecules 28, 4822-4823 (1995)). However, the yield of polyester (content of polyester in dried microorganisms) according to the processes described in Japanese Patent Laid Open Publication Nos. 93049/1993 and 265065/1995 is low, and thus there is demand for developments in a process for producing the copolymerized polyester P(3HB-co-3HH).

Summary of the Invention

The object of the present invention is to provide a polyester synthase gene, recombinant vectors containing the gene, transformants transformed with the recombinant vectors, and processes for producing polyester by use of the transformants.

As a result of their eager research, the present inventors succeeded in producing the polyester in high yield by cloning a polyester synthase gene and deleting one or both of open reading frames located upstream and downstream of said gene to arrive at the completion of the present invention.

That is, the present invention is a polyester synthase gene coding for a polypeptide containing the amino acid sequence of SEQ ID NO:2 or a sequence where in said amino acid sequence, one or more amino acids are deleted, replaced or added, said polypeptide bringing about polyester synthase activity. Said gene includes those containing e.g. the nucleotide sequence of SEQ ID NO:1.

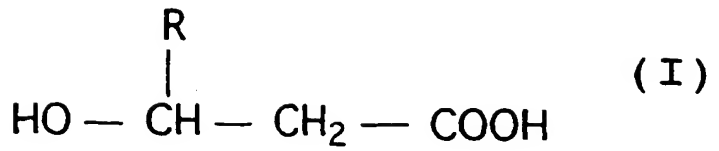
Further, the present invention is a gene expression cassette comprising said polyester synthase gene and either of open reading frames located upstream and downstream of said gene. In said gene expression cassette, the open reading frame located upstream of the polyester synthase gene includes those (e.g. SEQ ID NO:3) containing DNA coding for the amino acid sequence of SEQ ID NO:4, and the open reading frame located downstream of the polyester synthase gene includes those (e.g. SEQ ID NO:5) containing DNA coding for a polypeptide containing the amino acid sequence of SEQ ID NO:6 or a sequence where in said amino acid sequence, one or more amino acids are deleted, replaced or added, said polypeptide bringing about enoyl-CoA hydratase activity.

Even if one or more amino acids in the amino acid sequence of SEQ ID NO:2 have undergone mutations such as deletion, replacement, addition etc., DNA coding for a polypeptide containing said amino acid sequence is also contained in the gene of the present invention insofar as the polypeptide has polyester synthase activity. For example, DNA coding for the amino acid sequence of SEQ ID NO:2 where methionine at the first position is deleted is also contained in the gene of the present invention.

Further, the present invention is recombinant vectors comprising said polyester synthase gene or said gene expression cassette.

Further, the present invention is transformants transformed with said recombinant vectors.

Further, the present invention is processes for producing polyester, wherein said transformant is cultured in a medium, and polyester is recovered from the resulting culture. Examples of such polyester are copolymers (e.g. poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) random copolymers) of 3-hydroxyalkanoic acid represented by formula I:



(I)

wherein R represents a hydrogen atom or a C1 to C4 alkyl group.

Brief Description of the Drawing

FIG. 1 shows the structure of the gene of the present invention.

FIG. 2 is a photograph showing the result of SDS-polyacrylamide gel electrophoresis.

Detailed Description of the Invention

Hereinafter, the present invention is described in detail.

(1) Cloning of Polyester synthase gene

The polyester synthase gene of the present invention is separated from a microorganism belonging to the genus Aeromonas.

First, genomic DNA is isolated from a strain having the polyester synthase gene. Such a strain includes e.g. Aeromonas caviae.

Any known methods can be used for preparation of genomic DNA. For example, Aeromonas caviae is cultured in LB medium and then its genomic DNA is prepared by the hexadecyl trimethyl ammonium bromide method (Current Protocols in Molecular Biology, vol. 1, page 2.4.3., John Wiley & Sons Inc., 1994).

The DNA obtained in this manner is partially digested with a suitable restriction enzyme (e.g. Sau3AI, BamHI, BglII etc.) and then the DNA fragments are then dephosphorylated by treatment with alkaline phosphatase. It is ligated into a vector previously cleaved with a restriction enzyme (e.g. BamHI, BglII etc.) to prepare a library.

Phage or plasmid capable of autonomously replicating in host microorganisms is used as the vector. The phage vector includes e.g. EMBL3, M13, λgt11 etc., and the plasmid vector includes e.g. pBR322, pUC18, and pBluescript II (Stratagene). Vectors capable of autonomously replicating in 2 or more host cells such as E. coli and Bacillus brevis, as well as various shuttle vectors, can also be used. Such vectors are also cleaved with said restriction enzymes so that their fragment can be obtained.

Conventional DNA ligase is used to ligate the resulting DNA fragments into the vector fragment. The DNA fragments and the vector fragment are annealed and then ligated to produce a recombinant vector.

To introduce the recombinant vector into a host microorganism, any known methods can be used. For example, if the host microorganism is E. coli, the calcium method (Lederberg, E.M. et al., J. Bacteriol. 119, 1072 (1974)) and the electroporation method (Current Protocols in Molecular Biology, vol. 1, page 1.8.4 (1994)) can be used. If phage DNA is used, the in vitro packaging method (Current Protocols in Molecular Biology, vol. 1,, page 5.7.1 (1994)) etc. can be adopted. In the present invention, an in vitro packaging kit (Gigapack II, produced by Stratagene etc.) can also be used.

To obtain a DNA fragment containing the polyester synthase gene derived from Aeromonas caviae, a probe is then prepared. The amino acid sequences of some polyester synthase have already been known (Peoples, O.P. and Sinskey, A.J., J. Biol. Chem., 264, 15293 (1989); Huisman, G.W. et al., J. Biol. Chem., 266, 2191 (1991); Pieper, U. et al., FEMS Microbiol. Lett., 96, 73 (1992) etc.). Two conserved regions are selected from these amino acid sequences, and nucleotide sequences coding them are estimated to design oligonucleotides for use as primers. Examples of such oligonucleotides include, but are not limited to, the 2 oligonucleotides 5'-CC(C/G)CC(C/G)TGGATCAA(T/C)AAGT (T/A)(T/C)TA(T/C)ATC-3' (SEQ ID NO:7) and 5'-(G/C)AGCCA (G/C)GC(G/C)GTCCA(A/G)TC(G/C)GGCCACCA-3' (SEQ ID NO:8).

Polymerase chain reaction (PCR) (Molecular Cloning, vol. 2, page 14.2 (1989)) is carried out using these oligonucleotides as primers and the genomic DNA of Aeromonas caviae as a template. The partial fragment of polyester synthase gene is amplified by PCR.

Then, the partially amplified fragment thus obtained is labeled with a suitable reagent and used for colony hybridization of the above genomic DNA library (Current Protocols in Molecular Biology, vol. 1, page 6.0.3 (1994)).

The E. coli is screened by colony hybridization, and a plasmid is recovered from it using the alkaline method (Cur-

rent Protocols in Molecular Biology, vol. 1, page 1.6.1 (1994)), whereby a DNA fragment containing the polyester synthase gene is obtained.

The nucleotide sequence of said DNA fragment can be determined in e.g. an automatic nucleotide sequence analyzer such as 373A DNA sequencer (Applied Biosystems) using a known method such as the Sanger method (Molecular Cloning, vol. 2, page 13.3 (1989)).

The nucleotide sequence of the polyester synthase gene of the present invention is shown in SEQ ID NO:1, and the amino acid sequence encoded by said gene is shown in SEQ ID NO:2, where some amino acids may have undergone mutations such as deletion, replacement, addition etc. insofar as a polypeptide having said amino acid sequence brings about polyester synthase activity. Further, the gene of the present invention encompasses not only the nucleotide sequence coding for the amino acid sequence of SEQ ID NO:2 but also its degenerated isomers which except for degeneracy codons, code for the same polypeptide.

The above mutations such as deletion etc. can be induced by known site-directed mutagenesis (Current Protocols in Molecular Biology, vol., 1, page 8.1.1 (1994)).

After the nucleotide sequence was determined by the means described above, the gene of the present invention can be obtained by chemical synthesis or the PCR technique using genomic DNA as a template, or by hybridization using a DNA fragment having said nucleotide sequence as a probe.

(2) Preparation of Transformant

The transformant of the present invention is obtained by introducing the recombinant vector of the present invention into a host compatible with the expression vector used in constructing said recombinant vector.

The host is not particularly limited insofar as it can express the target gene. Examples are bacteria such as microorganisms belonging to the genus Alcaligenes, microorganisms belonging to the genus Pseudomonas, microorganisms belonging to the genus Bacillus, yeasts such as the genera Saccharomyces, Candida etc., and animal cells such as COS cells, CHO cells etc.

If bacteria such as microorganisms belonging to the genus Alcaligenes, microorganisms belonging to the genus Pseudomonas etc. are used as the host, the recombinant DNA of the present invention is preferably constituted such that it contains a promoter, the DNA of the present invention, and a transcription termination sequence so as to be capable of autonomous replication in the host. The expression vector includes pLA2917 (ATCC 37355) containing replication origin RK2 and pJRD215 (ATCC 37533) containing replication origin RSF1010, which are replicated and maintained in a broad range of hosts.

The promoter may be any one if it can be expressed in the host. Examples are promoters derived from E. coli, phage etc., such as trp promoter, lac promoter, P_L promoter, P_R promoter and T7 promoter. The method of introducing the recombinant DNA into bacteria includes e.g. a method using calcium ions (Current Protocols in Molecular Biology, vol. 1, page 1.8.1 (1994)) and the electroporation method (Current Protocols in Molecular Biology, vol. 1, page 1.8.4 (1994)).

If yeast is used as the host, expression vectors such as YEp13, YCp50 etc. are used. The promoter includes e.g. gal 1 promoter, gal 10 promoter etc. To method of introducing the recombinant DNA into yeast includes e.g. the electroporation method (Methods. Enzymol., 194, 182-187 (1990)), the spheroplast method (Proc. Natl. Acad. Sci. USA, 84, 1929-1933 (1978)), the lithium acetate method (J. Bacteriol., 153, 163-168 (1983)) etc.

If animal cells are used as the host, expression vectors such as pcDNAI, pcDNAI/Amp (produced by Invitrogene) etc. are used. The method of introducing the recombinant DNA into animal cells includes e.g. the electroporation method, potassium phosphate method etc.

The nucleotide sequence determined as described above contains the polyester synthase gene as well as a plurality of open reading frames (ORFs) upstream and downstream of it. That is, the polyester synthase gene forms an operon with at least 2 ORF's under the control of a single promoter region.

The ORF's which are located respectively upstream and downstream of the polyester synthase gene are referred to hereinafter as "ORF1" and "ORF3".

It is considered that ORF1 is an open reading frame of a gene involved in accumulating polyester in the microorganism or a gene in the polyester biosynthesis system. It was revealed that ORF3 is an open reading frame of a gene coding for enoyl-CoA hydratase (particularly (R)-specific enoyl-CoA hydratase) involved in biosynthesis of polyester.

As shown in FIG. 1, an EcoRI fragment carrying an expression regulatory region (expressed as "-35/-10" in FIG. 1A), the polyester synthase gene, ORF1, and ORF3 was cloned in the present invention (FIG. 1A). This fragment is designated EE32.

Then, a fragment (a gene expression cassette) is prepared by deleting ORF1 and/or ORF3 from EE32, and this cassette is introduced into a host whereby a transformant capable of efficiently producing polyester can be obtained.

In EE32, a restriction enzyme BglII sites are introduced into regions between the expression regulatory region and the translation initiation codon of ORF1 and between the translation termination codon of ORF1 and the translation ini-

tiation codon of the polyester synthase gene, and then ORF1 is deleted from EE32 by treatment with BglII (FIG. 1B). Similarly, a restriction enzyme BamHI sites is introduced into a region between the translation termination codon of the polyester synthase gene and ORF3, and then ORF3 is deleted by treatment with BamHI (FIG. 1C).

To delete both ORF1 and ORF3, EE32 may be subjected to the above operation of deleting ORF1 and ORF3 (FIG. 1D).

The restriction enzyme sites can be introduced by site-directed mutagenesis using synthetic oligonucleotides (Current Protocols in Molecular Biology, vol. 1, page 8.1.1 (1994)).

Each gene expression cassette thus obtained is inserted into said plasmid capable of expression (e.g. pJRD215 (ATCC 37533)) and the resulting recombinant vector is used to transform Alcaligenes eutrophus PHB-4 (DSM541) (strain deficient in the ability to synthesize polyester). The method for this transformation includes e.g. the calcium chloride method, rubidium chloride method, low pH method, in vitro packaging method, conjugation transfer method etc.

(3) Production of Polyester

The production of polyester is carried out by culturing the transformant of the present invention in a medium, forming and accumulating the polyester of the present invention in the microorganism or in the culture, and recovering the polyester from the cultured microorganism or from the culture.

A conventional method used for culturing the host is also used to culture the transformant of the present invention.

The medium for the transformant prepared from a microorganism belonging to the genus Alcaligenes or Pseudomonas as the host include a medium containing a carbon source assimilable by the microorganism, in which a nitrogen source, inorganic salts or another organic nutrition source has been limited, for example a medium in which the nutrition source has been limited to 0.01 to 0.1 %.

The carbon source is necessary for growth of the microorganism, and it is simultaneously a starting material of polyester. Examples are hydrocarbons such as glucose, fructose, sucrose, maltose etc. Further, fat and oil related substances having 2 or more carbon atoms can be used as the carbon source. The fat and oil related substances include natural fats and oils, such as corn oil, soybean oil, safflower oil, sunflower oil, olive oil, coconut oil, palm oil, rape oil, fish oil, whale oil, porcine oil and cattle oil, aliphatic acids such as acetic acid, propionic acid, butanoic acid, pentanoic acid, hexoic acid, octanoic acid, decanoic acid, lauric acid, oleic acid, palmitic acid, linolenic acid, linolic acid and myristic acid as well as esters thereof, alcohols such as ethanol, propanol, butanol, pentanol, hexanol, octanol, lauryl alcohol, oleyl alcohol and palmityl alcohol as well as esters thereof.

The nitrogen source includes e.g. ammonia, ammonium salts such as ammonium chloride, ammonium sulfate, ammonium phosphate etc., peptone, meat extract, yeast extract, corn steep liquor etc. The inorganic matter includes e.g. monopotassium phosphate, dipotassium phosphate, magnesium phosphate, magnesium sulfate, sodium chloride etc.

Culture is carried out usually under aerobic conditions with shaking at 25 to 37 °C for more than 24 hours (e.g. 1 to 7 days) after expression is induced. During culture, antibiotics such as ampicillin, kanamycin, antipyrine, tetracycline etc. may be added to the culture. Polyester is accumulated in the microorganism by culturing it, and the polyester is then recovered.

To culture the microorganism transformed with the expression vector using an inducible promoter, its inducer can also be added to the medium. For example, isopropyl-β-D-thiogalactopyranoside (IPTG), indoleacrylic acid (IAA) etc. can be added to the medium.

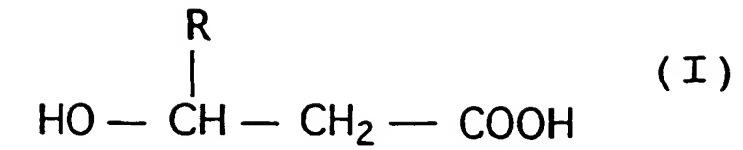
To culture the transformant from animal cells as the host, use is made of a medium such as RPMI-1640 or DMEM which may be supplemented with fetal bovine serum. Culture is carried out usually in the presence of 5 % CO_2 at 30 to 37°C for 14 to 28 days. During culture, antibiotics such as kanamycin, penicillin etc. may be added to the medium.

In the present invention, purification of polyester can be carried out e.g. as follows:

The transformant is recovered from the culture by centrifugation, then washed with distilled water and dried. Thereafter, the dried transformant is suspended in chloroform and heated to extract polyester from it. The residues are removed by filtration. Methanol is added to this chloroform solution to precipitate polyester. After the supernatant is removed by filtration or centrifugation, the precipitates are dried to give purified polyester.

The resulting polyester is confirmed to be the desired one in a usual manner e.g. by gas chromatography, nuclear magnetic resonance etc.

The gene of the present invention contains the polyester synthase gene isolated from Aeromonas caviae. This synthase can synthesize a copolymer (polyester) consisting of a monomer unit 3-hydroxyalkanoic acid represented by formula I:



(I)

wherein R represents a hydrogen atom or a C1 to C4 alkyl group. Said copolymer includes e.g. poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) random copolymer (P(3HB-co-3HH)) etc. and the transformant carrying said polyester synthase gene has the ability to produce P(3HB-co-3HH) with very high efficiency.

Conventionally, a process for producing poly-3-hydroxybutyrate (P(3HB)) or poly(3-hydroxybutyrate-co-3-hydroxyvalerate) random copolymer P(3HB-co-3HV) has been studied and developed, but such polyester has the problem in physical properties of inferior resistance to impact because it is a highly crystalline polymer.

Because degree of crystallinity is lowered by introducing 3-hydroxyhexanoate having 6 carbon atoms into a polymer chain, polyester acts as a flexible polymeric material which is also excellent in thermostability and formability, but conventional processes for producing P(3HB-co-3HH) by use of Aeromonas caviae (Japanese Patent Laid Open Publication Nos. 93049/1993 and 265065/1995) suffer from a low yield of polyester.

In the present invention, the P(3HB-co-3HH) copolyester can be produced in high yield.

Because the desired polyester can be obtained in a large amount using the above means, it can be used as a biodegradable material of yarn or film, various vessels etc. Further, the gene of the present invention can be used to breed a strain highly producing the P(3HB-co-3HH) copolymer polyester.

Examples

Hereinafter, the present invention is described in more detail with reference to the Examples which however are not intended to limit the scope of the present invention. (Example 1] Cloning of the Polyester synthase Gene from Aeromonas caviae

First, a genomic DNA library was prepared from Aeromonas caviae.

Aeromonas caviae FA440 was cultured overnight in 100 ml LB medium (1 % yeast extract, 0.5 % trypton, 0.5 % sodium chloride, 0.1 % glucose, pH 7.5) at 30 °C and then genomic DNA was obtained from the microorganism using the hexadecyl trimethyl ammonium bromide method (Current Protocols in Molecular Biology, vol. 1, page 2.4.3 (1994), John Wiley & Sons Inc.).

The resulting genomic DNA was partially digested with restriction enzyme Sau3AI. The vector plasmid used was cosmid vector pLA2917 (ATCC 37355).

This plasmid was cleaved with restriction enzyme BglII and dephosphorylated (Molecular Cloning, vol. 1, page 5.7.2 (1989), Cold Spring Harbor Laboratory) and then ligated into the partially digested genomic DNA fragment by use of DNA ligase.

E. coli S17-1 was transformed with this ligated DNA fragment by the in vitro packaging method (Current Protocols in Molecular Biology, vol. 1, page 5.7.2 (1994)) whereby a genomic DNA library from Aeromonas caviae was obtained.

To obtain a DNA fragment containing the polyester synthase gene from Aeromonas caviae, a probe was then prepared. Two well conserved regions were selected from known amino acid sequences of several polyester synthases, and nucleotide sequences coding for them were estimated, and 2 oligonucleotides 5'-CC(C/G)CC(C/G)TGGATCAA(T/C)AAGT (T/A)(T/C) TA(T/C)ATC-3' (SEQ ID NO:7) and 5'-(G/C)AGCCA(G/C)GC(G/C)GTCCA(A/G)TC(G/C)GGCCACCA-3' (SEQ ID NO:8) were synthesized.

The polyester synthase gene was partially amplified by PCR using these oligonucleotides as primers and the genomic DNA from Aeromonas caviae as a template. PCR was carried out using 30 cycles, each consisting of reaction at 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 60 seconds.

Then, this partially amplified fragment was labeled with digoxigenin using a DIG DNA labeling kit (Boehringer Mannheim) and used as a probe.

Using the probe thus obtained, E. coli carrying a plasmid containing the polyester synthase gene was isolated by colony hybridization from the genomic DNA library from Aeromonas caviae. By recovering the plasmid from the E. coli, a DNA fragment containing the polyester synthase gene was obtained.

The nucleotide sequence of a 3.2 kbp BglII-EcoRI fragment from this fragment was determined by the Sanger method.

As a result, the nucleotide sequence of the 3.2 kb fragment as shown in SEQ ID NOs:9 or 10 was determined.

By further examining homology to this nucleotide sequence, the polyester synthase gene containing the nucleotide sequence (1785 bp) of SEQ ID NO:1 could be identified in this 3.2 kbp nucleotide sequence.

It should be understood that insofar as the protein encoded by the polyester synthase gene of the present invention has the function of gene expression for polyester polymerization, the nucleotide sequence of said gene may have undergone mutations such as deletion, replacement, addition etc.

In a fragment having the nucleotide sequence of SEQ ID NO:9 or 10, a 405 bp gene (ORF3) and a transcription termination region located downstream of the above 1785 bp nucleotide sequence, as well as a 354 bp gene (ORF1) and an expression regulatory region located upstream thereof were identified. The nucleotide sequence of ORF1 is shown in SEQ ID NO:4; the nucleotide sequence of ORF3 in SEQ ID NO:5; and the amino acid sequence encoded by ORF3 in SEQ ID NO: 6.

ORF3 is an open reading frame of a gene coding for enoyl-CoA hydratase involved in biosynthesis of polyester. Insofar as a polypeptide having the amino acid sequence encoded by ORF3 has enoyl-CoA hydratase activity, particularly (R)-specific enoyl-CoA hydratase activity, said amino acid sequence may have undergone mutations such as deletion, replacement and addition of one or more amino acids.

In the nucleotide sequences of SEQ ID NOS:9 and 10, the expression regulatory region is located at the 1- to 383-positions and the transcription termination region at the 3010 to 3187- positions.

[Example 2] Preparation of Alcaligenes eutrophus Transformant

The BglII site of the BglII-EcoRI fragment containing this expression regulatory region, ORF1, the polyester synthase gene, ORF3, and the transcriptional termination region was made EcoRI-ended by use of an EcoRI linker whereby a 3.2 kb EcoRI-EcoRI fragment (EE32 fragment) was obtained. This fragment was inserted into plasmid pJRD215 (ATCC 37533) capable of expression in microorganisms belonging to the genus Alcaligenes, and the resulting recombinant plasmid was transformed into Alcaligenes eutrophus PHB-4 (DSM 541) (strain deficient in the ability to synthesize polyester) by the conjugation transfer method, as follows:

First, the recombinant plasmid was used to transform E.coli S17-1 by the calcium chloride method. The recombinant E.coli thus obtained and Alcaligenes eutrophus PHB-4 were cultured overnight in 1.5 ml LB medium at 30 °C, and the respective cultures, each 0.1 ml, were combined and cultured at 30 °C for 4 hours. This microbial mixture was plated on MBF agar medium (0.9 % disodium phosphate, 0.15 % monopotassium phosphate, 0.05 % ammonium chloride, 0.5 % fructose, 1.5 % agar, 0.3 mg/ml kanamycin) and cultured at 30 °C for 5 days.

Because Alcaligenes eutrophus PHB-4 is rendered resistant to kanamycin by transferring the plasmid in the recombinant E. coli into it, the colonies grown on the MBF agar medium are a transformant of Alcaligenes eutrophus. One colony was isolated from these colonies so that Alcaligenes eutrophus AC32 (referred to hereinafter as AC32) was obtained.

AC32 has been deposited as FERM BP-6038 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan.

A restriction enzyme BglII sites were introduced respectively into regions upstream and downstream of the ORF1 gene in the EE32 fragment by site-directed mutagenesis using a synthetic oligonucleotide (Current Protocols in Molecular Biology, vol. 1, page 8.1.1 (1994)), and an ORF1 gene-free fragment was obtained by deleting the BglII-BglII fragment from the EE32 fragment and then inserted into plasmid pJRD215. The resulting recombinant plasmid was used to transform Alcaligenes eutrophus PHB-4 by the conjugation transfer method described above. The resulting transformant is referred to hereinafter as AC321.

Similarly, a restriction enzyme BamHI sites were introduced respectively regions upstream and downstream of the ORF3 gene in the EE32 fragment by site-directed mutagenesis, and an ORF3 gene-free fragment was obtained by deleting the BamHI-BamHI fragment from the EE32 fragment and then inserted into plasmid pJRD215. The resulting recombinant plasmid was used to transform Alcaligenes eutrophus PHB-4 by the conjugation transfer method described above. The resulting transformant is referred to hereinafter as AC323.

Similarly, a restriction enzyme BglII sites were introduced respectively regions upstream and downstream of the ORF1 gene and a restriction enzyme BamHI sites were introduced respectively regions upstream and downstream of the ORF3 gene in the EE32 fragment, and a gene fragment free of both the ORF1 and ORF3 genes was obtained by deleting the BglII-BglII and BamHI-BamHI fragments from the EE32 fragment and then inserted into plasmid pJRD215. The resulting recombinant plasmid was used to transform Alcaligenes eutrophus PHB-4 by the conjugation transfer method described above. The resulting transformant is referred to hereinafter as AC3213.

Further, the polyester synthase gene was amplified by PCR using the EE32 fragment as a template, and the resulting amplification product was inserted into a region between an expression regulatory region and a transcription termination region in a known polyester biosynthesis operon derived from Alcaligenes eutrophus. PCR was carried out using 5'-AGTTCCCGCCTCGGGTGTGGGTGAA-3' (SEQ ID NO: 11) and 5'-GGCATATGCGCTCATGCGGCGTCCT-3' (SEQ ID NO: 12) as primers in 30 cycles each consisting of reaction at 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 60 seconds.

This DNA fragment was inserted into plasmid pJRD215, and the resulting plasmid was used to transform Alcali-

genes eutrophus PHB-4 by the conjugation transfer method described above. The resulting transformant is referred to hereinafter as AC29.

[Example 3] Synthesis of Polyester by Alcaligenes eutrophus Transformants

Each of Alcaligenes eutrophus H16, PHB-4, AC32, AC321, AC323, AC3213 and AC29 was inoculated into 95 ml MB medium (0.9 % disodium phosphate, 0.15 % monopotassium phosphate, 0.05 % ammonium chloride) containing 1 ml of 1 % sodium octanate and incubated in a flask at 30 °C. 0.2 g/L kanamycin was contained in the mediums for strains AC32, AC321, AC323, AC3213 and AC29. 12, 24, 36 and 48 hours thereafter, 1 ml of 1 % sodium octanate was added to each medium (total amount of sodium octanate added: 0.5 g) and the microorganisms were cultured for 72 hours.

Each of strains H16 and AC3213 was inoculated into the above MB medium to which 1% olive oil, palm oil, corn oil or oleic acid had been added, and each strain was cultured at 30 °C for 72 hours in a flask. 0.2 g/L kanamycin was contained in the mediums for strain AC3213.

Each of strains H16, AC32, AC321, AC323 and AC3213 was inoculated into the above MB medium to which 1% sodium heptanoate had been added, and each strain was cultured at 30 °C in a flask. 0.2 g/L kanamycin was contained in the mediums for strains AC32, AC321, AC323 and AC3213.

While 1 ml of 1% sodium heptanoate was added to each medium (total amount of sodium heptanoate added: 0.5 g) 12, 24, 36 and 48 hours thereafter, the microorganisms were cultured for 72 hours. 444

The microorganisms were recovered by centrifugation, washed with distilled water and lyophilized, and the weight of the dried microorganisms was determined. 2 ml sulfuric acid/methanol mixture (15 : 85) and 2 ml chloroform were added to 10-30 mg of the dried microorganism, and the sample was sealed and heated at 100 °C for 140 minutes whereby the polyester in the microorganisms was decomposed into methylester. 1 ml distilled water was added thereto and stirred vigorously. It was left and separated into 2 layers, and the lower organic layer was removed and analyzed for its components by capillary gas chromatography through a capillary column Neutra BOND-1 (column of 25 m in length, 0.25 mm in inner diameter and 0.4 μm in liquid film thickness, manufactured by GL Science) in Shimadzu GC-14A. The temperature was raised at a rate of 8 °C/min. from an initial temperature of 100 °C. The results are shown in Tables 1, 2 and 3.

Table 1

Synthesis of Polyester Using Octanoic Acid as Carbon Source				
Strain Used A. eutrophus	Weight of Dried Microorganism (g/l)	Content of Polyester in Dried Microorganism (weight-%)	Polyester Comp.	
			3HB	3HH
			(mole-%)	
H16	3.00	86	100	0
PHB-4	0.80	0	-	-
AC32	0.99	33	78	23
AC321	2.85	92	87	13
AC323	2.85	92	88	12
AC3213	3.64	96	85	15
AC29	3.20	94	92	8
3HB: 3-hydroxybutyrate, 3HH: 3-hydroxyhexanoate				

Table 2

Synthesis of Polyester Using Vegetable Oil or Oleic Acid as Carbon Source					
Strain Used A. eutrophus	Carbon Source	Weight of Dried Microorganism (g/l)	Content of Polyester in Dried Microorganism (weight-%)	Polyester Comp.	
				3HB	3HH
				(mole-%)	
H16	olive oil	4.27	79	100	0
	corn oil	3.57	81	100	0
	palm oil	4.13	79	100	0
	oleic acid	4.06	82	100	0
AC3213	olive oil	3.54	76	96	4
	corn oil	3.60	77	95	5
	palm oil	3.58	81	96	4
	oleic acid	2.22	70	96	4
3HB: 3-hydroxybutyrate, 3HH: 3-hydroxyhexanoate					

Table 3

Synthesis of Polyester Using Heptanoic Acid as Carbon Source					
Strain Used A. eutrophus	Weight of Dried Microorganism (g/l)	Content of Polyester in Dried Microorganism (weight-%)	Polyester Comp.		
			3HB	3HV	3HHp
			(mole-%)		
H16	2.50	60	50	50	0
AC32	0.77	7	30	67	5
AC321	1.67	55	46	52	2
AC323	1.27	40	48	45	7
AC3213	2.76	67	44	48	8
3HB: 3-hydroxybutyrate, 3HH: 3-hydroxyhexanoate, 3HHp: 3-hydroxyheptanoate					

As shown in Table 1, H16 (i.e. wild-type Alcaligenes eutrophus) synthesized a poly(3-hydroxybutyrate) homopolymer. This is because 3HH (3-hydroxyhexanoate) having 6 carbon atoms does not serve as a substrate for the polyester synthase possessed by H16. PHB-4 (i.e. the same strain as H16 but deficient in the ability to synthesize polyester) lacks the polyester synthase and thus does not accumulate polyester. AC32 prepared by introducing into PHB-4 the EE32 fragment containing the polyester synthase gene derived from Aeromonas caviae accumulated the poly(3-hydroxyburylate-co-3-hydroxyhexanoate) random copolymer (P(HB-co-3HH)) containing 22 mole-% 3HH (3-hydroxyhexanoate), and this copolymer accounted for 33 % by weight of the dried microorganism.

AC321, AC323 and AC3213 accumulated P(3HB-co-3HH) containing 12 to 15 mole-% 3HH, and the copolymer accounted for 92 to 96 % by weight of the dried microorganisms. As can be seen from these results, the ability of these strains to accumulate polyester was significantly improved by deleting the ORF1 gene and/or ORF3 gene.

P(3HB-co-3HH) was also accumulated in an amount of 94 % by weight of the microorganism even in the case of

AC29 carrying the polyester synthase gene derived from A. caviae whose expression regulatory region and transcriptional termination region had been replaced by those derived from Alcaligenes eutrophus, indicating that the yield of polyester was significantly improved even using the expression regulatory region and transcriptional termination region of different origin.

When AC3213 producing polyester in the highest yield was cultured using olive oil, corn oil or palm oil as a carbon source, the microorganism accumulated P(3HB-co-3HH) containing 4 to 5 mole-% 3HH, where the copolymer accounted for 76 to 81 % by weight of the microorganism, as shown in Table 2. Even if oleic acid as an fatty acid component contained most abundantly in vegetable oils was used as a carbon source, AC3213 accumulated P(3HB-co-3HH) containing 4 mole-% 3HH, where the copolymer accounted for 70 % by weight of the microorganism. Its corresponding wild strain H16 synthesized only poly(3-hydroxybutyrate) homopolymer under the same conditions.

Alcaligenes eutrophus FA440 is reported to have accumulated 8 % by weight of P(3HB-co-3HH) by use of palmitic acid as a carbon source (Japanese Patent Laid Open Publication No. 265065/1995). On the other hand, the transformant according to the present invention has accumulated 96 % by weight of P(3HB-co-3HH) by use of octanoic acid as a carbon source and 76 to 81 % by weight of P(3HB-co-3HH) by use of extremely cheap vegetable oils as a carbon source, so the comparison therebetween indicates that the method of synthesizing P(3HB-co-3HH) by the transformant used in the present example is an extremely superior method.

When heptanoic acid was used as a carbon source, H16, that is a wild strain of Alcaligenes eutrophus, synthesized poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer (P(3HB-co-3HV)). This is because 3HHp (3-hydroxyheptanoate) having 7 carbon atoms does not serve as a substrate for the polyester synthase possessed by H16, AC32, derived from PHB-4 by introduction of the EE32 fragment containing the polyester synthase gene derived from Aeromonas caviae, accumulated poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyheptanoate) terpolymer (P(3HB-co-3HV-co-3HHp)) containing 5 mole-% 3HHp, where this copolymer accounted for 7 % by weight of the dried microorganism.

Further, each of strains AC321, AC323 and AC3213 accumulated P(3HB-co-3HV-co-3HHp) containing 2 to 8 mole-% 3HHp where the copolymer accounted for 40 to 67 % by weight of the microorganisms, indicating that the yield of polyester was significantly improved by deleting the ORF1 gene and/or ORF3 gene (Table 3).

From these results, it is concluded that copolyesters consisting of 3-hydroxyalkanoic acid with 4 to 7 carbon atoms can be synthesized using the polyester synthase derived from Aeromonas caviae.

[Example 4] Identification of Functions of ORF3

The ORF3 gene was amplified by PCR using the EE32 fragment as a template and then inserted into a site downstream of T7 promoter in expression plasmid PET-3a (Novagene). PCR was carried out using 5'-GCCATATGAGCGCACAATCCCTGGAAGTAG-3' (SEQ ID NO:13) and 5'-CTGGGATCCGCCGGTGCTTAAGGCAGCTTG-3' (SEQ ID NO:14) as primers in 25 cycles each consisting of reaction at 95 °C for 60 seconds and 68 °C for 30 seconds. The resulting plasmid was used to transform E. coli BL21 (DE3) (Novagene). The resulting transformant is designated NB3.

NB3 was cultured in LB medium at 30 °C for 4 hours, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.4 mM to induce expression, and it was further cultured at 30 °C for 2 hours. The microorganism was recovered by centrifugation, disrupted by ultrasonication and centrifuged to give a soluble protein fraction.

As shown in Table 4, high enoyl-CoA hydratase activity was detected in the soluble fraction from the microorganism having the expression plasmid introduced into it.

Table 4

Specific Activity of Enoyl-CoA Hydratase in Soluble Protein Fraction

	(unit/mg protein)
E. coli BL21/PET-3a	0
E. coli NB3	1700

The enoyl-CoA hydratase activity was determined by measuring a change in absorbance (263 nm) due to double bond hydration, using crotonyl-CoA (Sigma) as substrate (concentration: 0.25 mM). No activity was detected in E. coli

into which the control plasmid PET-3a free of the ORF3 gene had been introduced.

Then, the enoyl-CoA hydratase protein was purified. A soluble protein fraction from NB3 was applied to an anion exchange column Q-Sepharose (Pharmacia) and eluted with a gradient of (0 to 1 M) NaCl, and a fraction with enoyl-CoA hydratase activity was collected. SDS-PAGE analysis indicated that the active fraction was homogenous in electrophoresis as shown in FIG. 2. In addition, about 3-fold specific activity could be attained as shown in Table 5.

Table 5

Specific Activity of Enoyl-CoA Hydratase

	(unit/mg protein)
E. coli NB3 soluble protein fraction	1700
anion exchange column elution fraction	5100

The N-terminal amino acid sequence of the enoyl-CoA hydratase protein thus purified was determined. As shown in Table 6, the determined amino acid sequence was the same except for Met in the initiation codon as the amino acid sequence deduced from the nucleotide sequence of the ORF3 gene.

Table 6

Comparison between Amino Acid Sequences

	(unit/mg protein)
N-terminal amino acid sequence of purified enoyl-CoA hydratase:	SAQSLEVGQKARLSKRFGAA (SEQ ID NO:15)
amino acid sequence deduced from ORF3 nucleotide sequence:	MSAQSLEVGQKARLSKRFGAA (SEQ ID NO:16)

From this, it could be confirmed that ORF3 codes for enoyl-CoA hydratase. It is considered that Met was released by post-translational modification.

Further, the stereospecificity of enoyl-CoA hydratase encoded by ORF3 was examined as follows:

By adding (S)-3-hydroxybutyryl-CoA dehydrogenase (Sigma) (final concentration: 0.2 unit/ml) and oxidized nicotinamide adenine dinucleotide (NAD+) (final concentration: 0.5 mM) to a reaction solution for activity measurement, (S)-3-hydroxybutyryl-CoA formed is oxidized to acetoacetyl-CoA by the action of the dehydrogenase if the enoyl-CoA hydratase is specific to the (S)-isomer. During this reaction, NAD+ is reduced to form NADH resulting in the generation of a specific absorption at 340 nm. If enoyl-CoA hydratase is specific to the (R)-isomer, NADH is not formed.

As shown in Table 7, the change in absorbance at 340 nm when enoyl-CoA hydratase encoded by ORF3 was used, was the same as in the case where enoyl-CoA hydratase was absent, but if commercially available (S)-specific enoyl-CoA hydratase (Sigma) was used, a change in absorbance due to formation of NADH was observed.

Table 7

Change in Absorbance at 340 nm after 1 Min.	
no addition of enoyl-CoA hydratase	0.045
ORF3-derived enoyl-CoA hydratase	0.047
(S)-isomer specific enoyl-CoA hydratase (Sigma)	0.146

From this result, it was made evident that the purified enoyl-CoA hydratase is specific to the (R)-isomer. Thus, it was found that ORF3 codes for (R)-isomer specific enoyl-CoA hydratase.

According to the present invention, there are provided a polyester synthase, a recombinant vector carrying the gene, a transformant carrying the recombinant vector and a process for producing polyester by use of the transformant.

The present invention is extremely useful in that the present gene codes for a polyester synthase capable of synthesizing polyester as a copolymer consisting of a monomer unit represented by 3-hydroxyalkanoic acid having 4 to 7 carbon atoms, and that the present process can synthesize a biodegradable plastic P(3HB-co-3HH) very efficiently which is excellent in thermostability and formability.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: POLYESTER SYNTHASE GENE AND PROCESS FOR PRODUCING POLYESTER

(iii) NUMBER OF SEQUENCES: 16

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: 97113932.4

(vi) PRIOR APPLICATION DATA:
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(B) FILING DATE: 14-AUG-1996

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: JP 199979/1997
(B) FILING DATE: 25-JUL-1997

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1785 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1782

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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ATG AGC CAA CCA TCT TAT GGC CCG CTG TTC GAG GCC CTG GCC CAC TAC      48
Met Ser Gln Pro Ser Tyr Gly Pro Leu Phe Glu Ala Leu Ala His Tyr
  1               5                  10                  15
AAT GAC AAG CTG CTG GCC ATG GCC AAG GCC CAG ACA GAG CGC ACC GCC      96
Asn Asp Lys Leu Leu Ala Met Ala Lys Ala Gln Thr Glu Arg Thr Ala
            20                  25                  30
CAG GCG CTG CTG CAG ACC AAT CTG GAC GAT CTG GGC CAG GTG CTG GAG     144
Gln Ala Leu Leu Gln Thr Asn Leu Asp Asp Leu Gly Gln Val Leu Glu
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          35                  40                  45
CAG GGC AGC CAG CAA CCC TGG CAG CTG ATC CAG GCC CAG ATG AAC TGG    192
Gln Gly Ser Gln Gln Pro Trp Gln Leu Ile Gln Ala Gln Met Asn Trp
     50                  55                  60
TGG CAG GAT CAG CTC AAG CTG ATG CAG CAC ACC CTG CTC AAA AGC GCA    240
Trp Gln Asp Gln Leu Lys Leu Met Gln His Thr Leu Leu Lys Ser Ala
 65                  70                  75                  80
GGC CAG CCG AGC GAG CCG GTG ATC ACC CCG GAG CGC AGC GAT CGC CGC    288
Gly Gln Pro Ser Glu Pro Val Ile Thr Pro Glu Arg Ser Asp Arg Arg
                 85                  90                  95
TTC AAG GCC GAG GCC TGG AGC GAA CAA CCC ATC TAT GAC TAC CTC AAG    336
Phe Lys Ala Glu Ala Trp Ser Glu Gln Pro Ile Tyr Asp Tyr Leu Lys
            100                 105                 110
CAG TCC TAC CTG CTC ACC GCC AGG CAC CTG CTG GCC TCG GTG GAT GCC    384
Gln Ser Tyr Leu Leu Thr Ala Arg His Leu Leu Ala Ser Val Asp Ala
        115                 120                 125
CTG GAG GGC GTC CCC CAG AAG AGC CGG GAG CGG CTG CGT TTC TTC ACC    432
Leu Glu Gly Val Pro Gln Lys Ser Arg Glu Arg Leu Arg Phe Phe Thr
    130                 135                 140
CGC CAG TAC GTC AAC GCC ATG GCC CCC AGC AAC TTC CTG GCC ACC AAC    480
Arg Gln Tyr Val Asn Ala Met Ala Pro Ser Asn Phe Leu Ala Thr Asn
145                 150                 155                 160
CCC GAG CTG CTC AAG CTG ACC CTG GAG TCC GAC GGC CAG AAC CTG GTG    528
Pro Glu Leu Leu Lys Leu Thr Leu Glu Ser Asp Gly Gln Asn Leu Val
                165                 170                 175
CGC GGA CTG GCC CTC TTG GCC GAG GAT CTG GAG CGC AGC GCC GAT CAG    576
Arg Gly Leu Ala Leu Leu Ala Glu Asp Leu Glu Arg Ser Ala Asp Gln
            180                 185                 190
CTC AAC ATC CGC CTG ACC GAC GAA TCC GCC TTC GAG CTC GGG CGG GAT    624
Leu Asn Ile Arg Leu Thr Asp Glu Ser Ala Phe Glu Leu Gly Arg Asp
        195                 200                 205
CTG GCC CTG ACC CCG GGC CGG GTG GTG CAG CGC ACC GAG CTC TAT GAG    672
Leu Ala Leu Thr Pro Gly Arg Val Val Gln Arg Thr Glu Leu Tyr Glu
    210                 215                 220
CTC ATT CAG TAC AGC CCG ACT ACC GAG ACG GTG GGC AAG ACA CCT GTG    720
Leu Ile Gln Tyr Ser Pro Thr Thr Glu Thr Val Gly Lys Thr Pro Val
225                 230                 235                 240
CTG ATA GTG CCG CCC TTC ATC AAC AAG TAC TAC ATC ATG GAC ATG CGG    768
Leu Ile Val Pro Pro Phe Ile Asn Lys Tyr Tyr Ile Met Asp Met Arg
                245                 250                 255
CCC CAG AAC TCC CTG GTC GCC TGG CTG GTC GCC CAG GGC CAG ACG GTA    816
Pro Gln Asn Ser Leu Val Ala Trp Leu Val Ala Gln Gly Gln Thr Val
            260                 265                 270
TTC ATG ATC TCC TGG CGC AAC CCG GGC GTG GCC CAG GCC CAA ATC GAT    864
Phe Met Ile Ser Trp Arg Asn Pro Gly Val Ala Gln Ala Gln Ile Asp
        275                 280                 285
CTC GAC GAC TAC GTG GTG GAT GGC GTC ATC GCC GCC CTG GAC GGC GTG    912
Leu Asp Asp Tyr Val Val Asp Gly Val Ile Ala Ala Leu Asp Gly Val
    290                 295                 300
GAG GCG GCC ACC GGC GAG CGG GAG GTG CAC GGC ATC GGC TAC TGC ATC    960
Glu Ala Ala Thr Gly Glu Arg Glu Val His Gly Ile Gly Tyr Cys Ile
305                 310                 315                 320
GGC GGC ACC GCC CTG TCG CTC GCC ATG GGC TGG CTG GCG GCG CGG CGC   1008
Gly Gly Thr Ala Leu Ser Leu Ala Met Gly Trp Leu Ala Ala Arg Arg
                325                 330                 335
CAG AAG CAG CGG GTG CGC ACC GCC ACC CTG TTC ACT ACC CTG CTG GAC   1056
Gln Lys Gln Arg Val Arg Thr Ala Thr Leu Phe Thr Thr Leu Leu Asp
            340                 345                 350
TTC TCC CAG CCC GGG GAG CTT GGC ATC TTC ATC CAC GAG CCC ATC ATA   1104
Phe Ser Gln Pro Gly Glu Leu Gly Ile Phe Ile His Glu Pro Ile Ile
        355                 360                 365
GCG GCG CTC GAG GCG CAA AAT GAG GCC AAG GGC ATC ATG GAC GGG CGC   1152
Ala Ala Leu Glu Ala Gln Asn Glu Ala Lys Gly Ile Met Asp Gly Arg
    370                 375                 380
CAG CTG GCG GTC TCC TTC AGC CTG CTG CGG GAG AAC AGC CTC TAC TGG   1200
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Gln Leu Ala Val Ser Phe Ser Leu Leu Arg Glu Asn Ser Leu Tyr Trp
385                 390                 395                 400
AAC TAC TAC ATC GAC AGC TAC CTC AAG GGT CAG AGC CCG GTG GCC TTC   1248
Asn Tyr Tyr Ile Asp Ser Tyr Leu Lys Gly Gln Ser Pro Val Ala Phe
                405                 410                 415
GAT CTG CTG CAC TGG AAC AGC GAC AGC ACC AAT GTG GCG GGC AAG ACC   1296
Asp Leu Leu His Trp Asn Ser Asp Ser Thr Asn Val Ala Gly Lys Thr
            420                 425                 430
CAC AAC AGC CTG CTG CGC CGT CTC TAC CTG GAG AAC CAG CTG GTG AAG   1344
His Asn Ser Leu Leu Arg Arg Leu Tyr Leu Glu Asn Gln Leu Val Lys
        435                 440                 445
GGG GAG CTC AAG ATC CGC AAC ACC CGC ATC GAT CTC GGC AAG GTG AAG   1392
Gly Glu Leu Lys Ile Arg Asn Thr Arg Ile Asp Leu Gly Lys Val Lys
    450                 455                 460
ACC CCT GTG CTG CTG GTG TCG GCG GTG GAC GAT CAC ATC GCC CTC TGG   1440
Thr Pro Val Leu Leu Val Ser Ala Val Asp Asp His Ile Ala Leu Trp
465                 470                 475                 480
CAG GGC ACC TGG CAG GGC ATG AAG CTG TTT GGC GGG GAG CAG CGC TTC   1488
Gln Gly Thr Trp Gln Gly Met Lys Leu Phe Gly Gly Glu Gln Arg Phe
                485                 490                 495
CTC CTG GCG GAG TCC GGC CAC ATC GCC GGC ATC ATC AAC CCG CCG GCC   1536
Leu Leu Ala Glu Ser Gly His Ile Ala Gly Ile Ile Asn Pro Pro Ala
            500                 505                 510
GCC AAC AAG TAC GGC TTC TGG CAC AAC GGG GCC GAG GCC GAG AGC CCG   1584
Ala Asn Lys Tyr Gly Phe Trp His Asn Gly Ala Glu Ala Glu Ser Pro
        515                 520                 525
GAG AGC TGG CTG GCA GGG GCG ACG CAC CAG GGC GGC TCC TGG TGG CCC   1632
Glu Ser Trp Leu Ala Gly Ala Thr His Gln Gly Gly Ser Trp Trp Pro
    530                 535                 540
GAG ATG ATG GGC TTT ATC CAG AAC CGT GAC GAA GGG TCA GAG CCC GTC   1680
Glu Met Met Gly Phe Ile Gln Asn Arg Asp Glu Gly Ser Glu Pro Val
545                 550                 555                 560
CCC GCG CGG GTC CCG GAG GAA GGG CTG GCC CCC GCC CCC GGC CAC TAT   1728
Pro Ala Arg Val Pro Glu Glu Gly Leu Ala Pro Ala Pro Gly His Tyr
                565                 570                 575
GTC AAG GTG CGG CTC AAC CCC GTG TTT GCC TGC CCA ACA GAG GAG GAC   1776
Val Lys Val Arg Leu Asn Pro Val Phe Ala Cys Pro Thr Glu Glu Asp
            580                 585                 590
GCC GCA TGA                                                       1785
Ala Ala
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 594 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Met Ser Gln Pro Ser Tyr Gly Pro Leu Phe Glu Ala Leu Ala His Tyr
 1               5                  10                  15

Asn Asp Lys Leu Leu Ala Met Ala Lys Ala Gln Thr Glu Arg Thr Ala
            20                  25                  30
Gln Ala Leu Leu Gln Thr Asn Leu Asp Asp Leu Gly Gln Val Leu Glu
        35                  40                  45
Gln Gly Ser Gln Gln Pro Trp Gln Leu Ile Gln Ala Gln Met Asn Trp
    50                  55                  60
Trp Gln Asp Gln Leu Lys Leu Met Gln His Thr Leu Leu Lys Ser Ala
65                  70                  75                  80
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Gly Gln Pro Ser Glu Pro Val Ile Thr Pro Glu Arg Ser Asp Arg Arg
                85                  90                  95
Phe Lys Ala Glu Ala Trp Ser Glu Gln Pro Ile Tyr Asp Tyr Leu Lys
            100                 105                 110
Gln Ser Tyr Leu Leu Thr Ala Arg His Leu Leu Ala Ser Val Asp Ala
        115                 120                 125
Leu Glu Gly Val Pro Gln Lys Ser Arg Glu Arg Leu Arg Phe Phe Thr
    130                 135                 140
Arg Gln Tyr Val Asn Ala Met Ala Pro Ser Asn Phe Leu Ala Thr Asn
145                 150                 155                 160
Pro Glu Leu Leu Lys Leu Thr Leu Glu Ser Asp Gly Gln Asn Leu Val
                165                 170                 175
Arg Gly Leu Ala Leu Leu Ala Glu Asp Leu Glu Arg Ser Ala Asp Gln
            180                 185                 190
Leu Asn Ile Arg Leu Thr Asp Glu Ser Ala Phe Glu Leu Gly Arg Asp
        195                 200                 205
Leu Ala Leu Thr Pro Gly Arg Val Val Gln Arg Thr Glu Leu Tyr Glu
    210                 215                 220
Leu Ile Gln Tyr Ser Pro Thr Thr Glu Thr Val Gly Lys Thr Pro Val
225                 230                 235                 240
Leu Ile Val Pro Pro Phe Ile Asn Lys Tyr Tyr Ile Met Asp Met Arg
                245                 250                 255
Pro Gln Asn Ser Leu Val Ala Trp Leu Val Ala Gln Gly Gln Thr Val
            260                 265                 270
Phe Met Ile Ser Trp Arg Asn Pro Gly Val Ala Gln Ala Gln Ile Asp
        275                 280                 285
Leu Asp Asp Tyr Val Val Asp Gly Val Ile Ala Ala Leu Asp Gly Val
    290                 295                 300
Glu Ala Ala Thr Gly Glu Arg Glu Val His Gly Ile Gly Tyr Cys Ile
305                 310                 315                 320
Gly Gly Thr Ala Leu Ser Leu Ala Met Gly Trp Leu Ala Ala Arg Arg
                325                 330                 335
Gln Lys Gln Arg Val Arg Thr Ala Thr Leu Phe Thr Thr Leu Leu Asp
            340                 345                 350
Phe Ser Gln Pro Gly Glu Leu Gly Ile Phe Ile His Glu Pro Ile Ile
        355                 360                 365
Ala Ala Leu Glu Ala Gln Asn Glu Ala Lys Gly Ile Met Asp Gly Arg
    370                 375                 380
Gln Leu Ala Val Ser Phe Ser Leu Leu Arg Glu Asn Ser Leu Tyr Trp
385                 390                 395                 400
Asn Tyr Tyr Ile Asp Ser Tyr Leu Lys Gly Gln Ser Pro Val Ala Phe
                405                 410                 415
Asp Leu Leu His Trp Asn Ser Asp Ser Thr Asn Val Ala Gly Lys Thr
            420                 425                 430
His Asn Ser Leu Leu Arg Arg Leu Tyr Leu Glu Asn Gln Leu Val Lys
        435                 440                 445
Gly Glu Leu Lys Ile Arg Asn Thr Arg Ile Asp Leu Gly Lys Val Lys
    450                 455                 460
Thr Pro Val Leu Leu Val Ser Ala Val Asp Asp His Ile Ala Leu Trp
465                 470                 475                 480
Gln Gly Thr Trp Gln Gly Met Lys Leu Phe Gly Gly Glu Gln Arg Phe
                485                 490                 495
Leu Leu Ala Glu Ser Gly His Ile Ala Gly Ile Ile Asn Pro Pro Ala
            500                 505                 510
Ala Asn Lys Tyr Gly Phe Trp His Asn Gly Ala Glu Ala Glu Ser Pro
        515                 520                 525
Glu Ser Trp Leu Ala Gly Ala Thr His Gln Gly Gly Ser Trp Trp Pro
    530                 535                 540
Glu Met Met Gly Phe Ile Gln Asn Arg Asp Glu Gly Ser Glu Pro Val
545                 550                 555                 560
Pro Ala Arg Val Pro Glu Glu Gly Leu Ala Pro Ala Pro Gly His Tyr
                565                 570                 575
Val Lys Val Arg Leu Asn Pro Val Phe Ala Cys Pro Thr Glu Glu Asp
            580                 585                 590
Ala Ala
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 354 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..351

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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ATG ATG AAT ATG GAC GTG ATC AAG AGC TTT ACC GAG CAG ATG CAA GGC   û@48
Met Met Asn Met Asp Val Ile Lys Ser Phe Thr Glu Gln Met Gln Gly
  1               5                  10                  15
TTC GCC GCC CCC CTC ACC CGC TAC AAC CAG CTG CTG GCC AGC AAC ATC   û@96
Phe Ala Ala Pro Leu Thr Arg Tyr Asn Gln Leu Leu Ala Ser Asn Ile
            20                  25                  30
GAA CAG CTG ACC CGG TTG CAG CTG GCC TCC GCC AAC GCC TAC GCC GAA    144
Glu Gln Leu Thr Arg Leu Gln Leu Ala Ser Ala Asn Ala Tyr Ala Glu
        35                  40                  45
CTG GGC CTC AAC CAG TTG CAG GCC GTG AGC AAG GTG CAG GAC ACC CAG    192
Leu Gly Leu Asn Gln Leu Gln Ala Val Ser Lys Val Gln Asp Thr Gln
    50                  55                  60
AGC CTG GCG GCC CTG GGC ACA GTG CAA CTG GAG ACC GCC AGC CAG CTC    240
Ser Leu Ala Ala Leu Gly Thr Val Gln Leu Glu Thr Ala Ser Gln Leu
65                  70                  75                  80
TCC CGC CAG ATG CTG GAT GAC ATC CAG AAG CTG AGC GCC CTC GGC CAG    288
Ser Arg Gln Met Leu Asp Asp Ile Gln Lys Leu Ser Ala Leu Gly Gln
                85                  90                  95
CAG TTC AAG GAA GAG CTG GAT GTC CTG ACC GCA GAC GGC ATC AAG AAA    336
Gln Phe Lys Glu Glu Leu Asp Val Leu Thr Ala Asp Gly Ile Lys Lys
            100                 105                 110
AGC ACG GGC AAG GCC TGA                                        354
Ser Thr Gly Lys Ala
        115
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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 117 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Met Met Asn Met Asp Val Ile Lys Ser Phe Thr Glu Gln Met Gln Gly
  1               5                  10                  15
Phe Ala Ala Pro Leu Thr Arg Tyr Asn Gln Leu Leu Ala Ser Asn Ile
            20                  25                  30
Glu Gln Leu Thr Arg Leu Gln Leu Ala Ser Ala Asn Ala Tyr Ala Glu
        35                  40                  45
Leu Gly Leu Asn Gln Leu Gln Ala Val Ser Lys Val Gln Asp Thr Gln
    50                  55                  60
Ser Leu Ala Ala Leu Gly Thr Val Gln Leu Glu Thr Ala Ser Gln Leu
```

```
 65                  70                  75                  80
Ser Arg Gln Met Leu Asp Asp Ile Gln Lys Leu Ser Ala Leu Gly Gln
                85                  90                  95
Gln Phe Lys Glu Glu Leu Asp Val Leu Thr Ala Asp Gly Ile Lys Lys
            100                 105                 110
Ser Thr Gly Lys Ala
        115
```

2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 405 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..402

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
ATG AGC GCA CAA TCC CTG GAA GTA GGC CAG AAG GCC CGT CTC AGC AAG      48
Met Ser Ala Gln Ser Leu Glu Val Gly Gln Lys Ala Arg Leu Ser Lys
  1               5                  10                  15
CGG TTC GGG GCG GCG GAG GTA GCC GCC TTC GCC GCG CTC TCG GAG GAC      96
Arg Phe Gly Ala Ala Glu Val Ala Ala Phe Ala Ala Leu Ser Glu Asp
            20                  25                  30
TTC AAC CCC CTG CAC CTG GAC CCG GCC TTC GCC GCC ACC ACG GCG TTC     144
Phe Asn Pro Leu His Leu Asp Pro Ala Phe Ala Ala Thr Thr Ala Phe
        35                  40                  45
GAG CGG CCC ATA GTC CAC GGC ATG CTG CTC GCC AGC CTC TTC TCC GGG     192
Glu Arg Pro Ile Val His Gly Met Leu Leu Ala Ser Leu Phe Ser Gly
    50                  55                  60
CTG CTG GGC CAG CAG TTG CCG GGC AAG GGG AGC ATC TAT CTG GGT CAA     240
Leu Leu Gly Gln Gln Leu Pro Gly Lys Gly Ser Ile Tyr Leu Gly Gln
65                  70                  75                  80
AGC CTC AGC TTC AAG CTG CCG GTC TTT GTC GGG GAC GAG GTG ACG GCC     288
Ser Leu Ser Phe Lys Leu Pro Val Phe Val Gly Asp Glu Val Thr Ala
                85                  90                  95
GAG GTG GAG GTG ACC GCC CTT CGC GAG GAC AAG CCC ATC GCC ACC CTG     336
Glu Val Glu Val Thr Ala Leu Arg Glu Asp Lys Pro Ile Ala Thr Leu
            100                 105                 110
ACC ACC CGC ATC TTC ACC CAA GGC GGC GCC CTC GCC GTG ACG GGG GAA     384
Thr Thr Arg Ile Phe Thr Gln Gly Gly Ala Leu Ala Val Thr Gly Glu
        115                 120                 125
GCC GTG GTC AAG CTG CCT TAA                                        405
Ala Val Val Lys Leu Pro
    130
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 134 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
Met Ser Ala Gln Ser Leu Glu Val Gly Gln Lys Ala Arg Leu Ser Lys
 1               5                  10                  15
Arg Phe Gly Ala Ala Glu Val Ala Ala Phe Ala Ala Leu Ser Glu Asp
            20                  25                  30
Phe Asn Pro Leu His Leu Asp Pro Ala Phe Ala Ala Thr Thr Ala Phe
        35                  40                  45
Glu Arg Pro Ile Val His Gly Met Leu Leu Ala Ser Leu Phe Ser Gly
    50                  55                  60
Leu Leu Gly Gln Gln Leu Pro Gly Lys Gly Ser Ile Tyr Leu Gly Gln
65                  70                  75                  80
Ser Leu Ser Phe Lys Leu Pro Val Phe Val Gly Asp Glu Val Thr Ala
                85                  90                  95
Glu Val Glu Val Thr Ala Leu Arg Glu Asp Lys Pro Ile Ala Thr Leu
            100                 105                 110
Thr Thr Arg Ile Phe Thr Gln Gly Gly Ala Leu Ala Val Thr Gly Glu
        115                 120                 125
Ala Val Val Lys Leu Pro
    130
```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CCSCCSTGGA TCAAYAAGTW YTAYATC 27

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

SAGCCASGCS GTCCARTCSG GCCACCA 27

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3187 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 384..734

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 830..2611

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

```
AGATCTGGAC CGGGGTGCTG GCCTGGGCCA CGCCGGCGAG GGCCAGCGCG GAGCAACCGA   60
GCAGCAGGGC GAGAGGTTTC ATCGGGATTC CTTGGCAGTC TGAATGACGT GCCAGCCTAT  120
CAGCGCGGCG CCGGTGCGGC GAGGGCGCGC CGGACCCAGT GCGTCACCTC TCGTCTGATC  180
CGCCTCCCTC GACGGGCGTC GCTGACAAAA AAATTCAAAC AGAAATTAAC ATTTATGTCA  240
TTTACACCAA ACCGCATTTG GTTGCAGAAT GCTCAAACGT GTGTTTGAAC AGAGCAAGCA  300
ACACGTAAAC AGGGATGACA TGCAGTACCC GTAAGAAGGG CCGATTGGCC CACAACAACA  360
CTGTTCTGCC GAACTGGAGA CCG ATG ATG AAT ATG GAC GTG ATC AAG AGC.       410
                          Met Met Asn Met Asp Val Ile Lys Ser
                            1               5
TTT ACC GAG CAG ATG CAA GGC TTC GCC GCC CCC CTC ACC CGC TAC AAC     458
Phe Thr Glu Gln Met Gln Gly Phe Ala Ala Pro Leu Thr Arg Tyr Asn
 10                  15                  20                  25
CAG CTG CTG GCC AGC AAC ATC GAA CAG CTG ACC CGG TTG CAG CTG GCC     506
Gln Leu Leu Ala Ser Asn Ile Glu Gln Leu Thr Arg Leu Gln Leu Ala
                 30                  35                  40
TCC GCC AAC GCC TAC GCC GAA CTG GGC CTC AAC CAG TTG CAG GCC GTG     554
Ser Ala Asn Ala Tyr Ala Glu Leu Gly Leu Asn Gln Leu Gln Ala Val
             45                  50                  55
AGC AAG GTG CAG GAC ACC CAG AGC CTG GCG GCC CTG GGC ACA GTG CAA     602
Ser Lys Val Gln Asp Thr Gln Ser Leu Ala Ala Leu Gly Thr Val Gln
         60                  65                  70
CTG GAG ACC GCC AGC CAG CTC TCC CGC CAG ATG CTG GAT GAC ATC CAG     650
Leu Glu Thr Ala Ser Gln Leu Ser Arg Gln Met Leu Asp Asp Ile Gln
     75                  80                  85
AAG CTG AGC GCC CTC GGC CAG CAG TTC AAG GAA GAG CTG GAT GTC CTG     698
Lys Leu Ser Ala Leu Gly Gln Gln Phe Lys Glu Glu Leu Asp Val Leu
 90                  95                 100                 105
ACC GCA GAC GGC ATC AAG AAA AGC ACG GGC AAG GCC TGATAACCCC          744
Thr Ala Asp Gly Ile Lys Lys Ser Thr Gly Lys Ala
                110                 115
TGGCTGCCCG TTCGGGCAGC CACATCTCCC CATGACTCGA CGCTACGGGC TAGTTCCCGC   804
CTCGGGTGTG GGTGAAGGAG AGCAC ATG AGC CAA CCA TCT TAT GGC CCG CTG      856
                           Met Ser Gln Pro Ser Tyr Gly Pro Leu
                             1               5
TTC GAG GCC CTG GCC CAC TAC AAT GAC AAG CTG CTG GCC ATG GCC AAG     904
Phe Glu Ala Leu Ala His Tyr Asn Asp Lys Leu Leu Ala Met Ala Lys
 10                  15                  20                  25
GCC CAG ACA GAG CGC ACC GCC CAG GCG CTG CTG CAG ACC AAT CTG GAC     952
Ala Gln Thr Glu Arg Thr Ala Gln Ala Leu Leu Gln Thr Asn Leu Asp
                 30                  35                  40
GAT CTG GGC CAG GTG CTG GAG CAG GGC AGC CAG CAA CCC TGG CAG CTG    1000
Asp Leu Gly Gln Val Leu Glu Gln Gly Ser Gln Gln Pro Trp Gln Leu
             45                  50                  55
ATC CAG GCC CAG ATG AAC TGG TGG CAG GAT CAG CTC AAG CTG ATG CAG    1048
Ile Gln Ala Gln Met Asn Trp Trp Gln Asp Gln Leu Lys Leu Met Gln
         60                  65                  70
CAC ACC CTG CTC AAA AGC GCA GGC CAG CCG AGC GAG CCG GTG ATC ACC    1096
His Thr Leu Leu Lys Ser Ala Gly Gln Pro Ser Glu Pro Val Ile Thr
     75                  80                  85
CCG GAG CGC AGC GAT CGC CGC TTC AAG GCC GAG GCC TGG AGC GAA CAA    1144
Pro Glu Arg Ser Asp Arg Arg Phe Lys Ala Glu Ala Trp Ser Glu Gln
 90                  95                 100                 105
CCC ATC TAT GAC TAC CTC AAG CAG TCC TAC CTG CTC ACC GCC AGG CAC    1192
Pro Ile Tyr Asp Tyr Leu Lys Gln Ser Tyr Leu Leu Thr Ala Arg His
                110                 115                 120
CTG CTG GCC TCG GTG GAT GCC CTG GAG GGC GTC CCC CAG AAG AGC CGG    1240
```

```
Leu Leu Ala Ser Val Asp Ala Leu Glu Gly Val Pro Gln Lys Ser Arg
                125                 130                 135
GAG CGG CTG CGT TTC TTC ACC CGC CAG TAC GTC AAC GCC ATG GCC CCC   1288
Glu Arg Leu Arg Phe Phe Thr Arg Gln Tyr Val Asn Ala Met Ala Pro
        140                 145                 150
AGC AAC TTC CTG GCC ACC AAC CCC GAG CTG CTC AAG CTG ACC CTG GAG   1336
Ser Asn Phe Leu Ala Thr Asn Pro Glu Leu Leu Lys Leu Thr Leu Glu
    155                 160                 165
TCC GAC GGC CAG AAC CTG GTG CGC GGA CTG GCC CTC TTG GCC GAG GAT   1384
Ser Asp Gly Gln Asn Leu Val Arg Gly Leu Ala Leu Leu Ala Glu Asp
170                 175                 180                 185
CTG GAG CGC AGC GCC GAT CAG CTC AAC ATC CGC CTG ACC GAC GAA TCC   1432
Leu Glu Arg Ser Ala Asp Gln Leu Asn Ile Arg Leu Thr Asp Glu Ser
                190                 195                 200
GCC TTC GAG CTC GGG CGG GAT CTG GCC CTG ACC CCG GGC CGG GTG GTG   1480
Ala Phe Glu Leu Gly Arg Asp Leu Ala Leu Thr Pro Gly Arg Val Val
            205                 210                 215
CAG CGC ACC GAG CTC TAT GAG CTC ATT CAG TAC AGC CCG ACT ACC GAG   1528
Gln Arg Thr Glu Leu Tyr Glu Leu Ile Gln Tyr Ser Pro Thr Thr Glu
        220                 225                 230
ACG GTG GGC AAG ACA CCT GTG CTG ATA GTG CCG CCC TTC ATC AAC AAG   1576
Thr Val Gly Lys Thr Pro Val Leu Ile Val Pro Pro Phe Ile Asn Lys
    235                 240                 245
TAC TAC ATC ATG GAC ATG CGG CCC CAG AAC TCC CTG GTC GCC TGG CTG   1624
Tyr Tyr Ile Met Asp Met Arg Pro Gln Asn Ser Leu Val Ala Trp Leu
250                 255                 260                 265
GTC GCC CAG GGC CAG ACG GTA TTC ATG ATC TCC TGG CGC AAC CCG GGC   1672
Val Ala Gln Gly Gln Thr Val Phe Met Ile Ser Trp Arg Asn Pro Gly
                270                 275                 280
GTG GCC CAG GCC CAA ATC GAT CTC GAC GAC TAC GTG GTG GAT GGC GTC   1720
Val Ala Gln Ala Gln Ile Asp Leu Asp Asp Tyr Val Val Asp Gly Val
            285                 290                 295
ATC GCC GCC CTG GAC GGC GTG GAG GCG GCC ACC GGC GAG CGG GAG GTG   1768
Ile Ala Ala Leu Asp Gly Val Glu Ala Ala Thr Gly Glu Arg Glu Val
        300                 305                 310
CAC GGC ATC GGC TAC TGC ATC GGC GGC ACC GCC CTG TCG CTC GCC ATG   1816
His Gly Ile Gly Tyr Cys Ile Gly Gly Thr Ala Leu Ser Leu Ala Met
    315                 320                 325
GGC TGG CTG GCG GCG CGG CGC CAG AAG CAG CGG GTG CGC ACC GCC ACC   1864
Gly Trp Leu Ala Ala Arg Arg Gln Lys Gln Arg Val Arg Thr Ala Thr
330                 335                 340                 345
CTG TTC ACT ACC CTG CTG GAC TTC TCC CAG CCC GGG GAG CTT GGC ATC   1912
Leu Phe Thr Thr Leu Leu Asp Phe Ser Gln Pro Gly Glu Leu Gly Ile
                350                 355                 360
TTC ATC CAC GAG CCC ATC ATA GCG GCG CTC GAG GCG CAA AAT GAG GCC   1960
Phe Ile His Glu Pro Ile Ile Ala Ala Leu Glu Ala Gln Asn Glu Ala
            365                 370                 375
AAG GGC ATC ATG GAC GGG CGC CAG CTG GCG GTC TCC TTC AGC CTG CTG   2008
Lys Gly Ile Met Asp Gly Arg Gln Leu Ala Val Ser Phe Ser Leu Leu
        380                 385                 390
CGG GAG AAC AGC CTC TAC TGG AAC TAC TAC ATC GAC AGC TAC CTC AAG   2056
Arg Glu Asn Ser Leu Tyr Trp Asn Tyr Tyr Ile Asp Ser Tyr Leu Lys
    395                 400                 405
GGT CAG AGC CCG GTG GCC TTC GAT CTG CTG CAC TGG AAC AGC GAC AGC   2104
Gly Gln Ser Pro Val Ala Phe Asp Leu Leu His Trp Asn Ser Asp Ser
410                 415                 420                 425
ACC AAT GTG GCG GGC AAG ACC CAC AAC AGC CTG CTG CGC CGT CTC TAC   2152
Thr Asn Val Ala Gly Lys Thr His Asn Ser Leu Leu Arg Arg Leu Tyr
                430                 435                 440
CTG GAG AAC CAG CTG GTG AAG GGG GAG CTC AAG ATC CGC AAC ACC CGC   2200
Leu Glu Asn Gln Leu Val Lys Gly Glu Leu Lys Ile Arg Asn Thr Arg
            445                 450                 455
ATC GAT CTC GGC AAG GTG AAG ACC CCT GTG CTG CTG GTG TCG GCG GTG   2248
Ile Asp Leu Gly Lys Val Lys Thr Pro Val Leu Leu Val Ser Ala Val
        460                 465                 470
```

```
GAC GAT CAC ATC GCC CTC TGG CAG GGC ACC TGG CAG GGC ATG AAG CTG   2296
Asp Asp His Ile Ala Leu Trp Gln Gly Thr Trp Gln Gly Met Lys Leu
    475                 480                 485
TTT GGC GGG GAG CAG CGC TTC CTC CTG GCG GAG TCC GGC CAC ATC GCC   2344
Phe Gly Gly Glu Gln Arg Phe Leu Leu Ala Glu Ser Gly His Ile Ala
490                 495                 500                 505
GGC ATC ATC AAC CCG CCG GCC GCC AAC AAG TAC GGC TTC TGG CAC AAC   2392
Gly Ile Ile Asn Pro Pro Ala Ala Asn Lys Tyr Gly Phe Trp His Asn
                510                 515                 520
GGG GCC GAG GCC GAG AGC CCG GAG AGC TGG CTG GCA GGG GCG ACG CAC   2440
Gly Ala Glu Ala Glu Ser Pro Glu Ser Trp Leu Ala Gly Ala Thr His
            525                 530                 535
CAG GGC GGC TCC TGG TGG CCC GAG ATG ATG GGC TTT ATC CAG AAC CGT   2488
Gln Gly Gly Ser Trp Trp Pro Glu Met Met Gly Phe Ile Gln Asn Arg
        540                 545                 550
GAC GAA GGG TCA GAG CCC GTC CCC GCG CGG GTC CCG GAG GAA GGG CTG   2536
Asp Glu Gly Ser Glu Pro Val Pro Ala Arg Val Pro Glu Glu Gly Leu
    555                 560                 565
GCC CCC GCC CCC GGC CAC TAT GTC AAG GTG CGG CTC AAC CCC GTG TTT   2584
Ala Pro Ala Pro Gly His Tyr Val Lys Val Arg Leu Asn Pro Val Phe
570                 575                 580                 585
GCC TGC CCA ACA GAG GAG GAC GCC GCA TGAGCGCACA ATCCCTGGAA         2631
Ala Cys Pro Thr Glu Glu Asp Ala Ala
                590
GTAGGCCAGA AGGCCCGTCT CAGCAAGCGG TTCGGGGCGG CGGAGGTAGC CGCCTTCGCC 2691
GCGCTCTCGG AGGACTTCAA CCCCCTGCAC CTGGACCCGG CCTTCGCCGC CACCACGGCG 2751
TTCGAGCGGC CCATAGTCCA CGGCATGCTG CTCGCCAGCC TCTTCTCCGG GCTGCTGGGC 2811
CAGCAGTTGC CGGGCAAGGG GAGCATCTAT CTGGGTCAAA GCCTCAGCTT CAAGCTGCCG 2871
GTCTTTGTCG GGGACGAGGT GACGGCCGAG GTGGAGGTGA CCGCCCTTCG CGAGGACAAG 2931
CCCATCGCCA CCCTGACCAC CCGCATCTTC ACCCAAGGCG GCGCCCTCGC CGTGACGGGG 2991
GAAGCCGTGG TCAAGCTGCC TTAAGCACCG GCGGCACGCA GGCACAATCA GCCCGGCCCC 3051
TGCCGGGCTG ATTGTTCTCC CCCGCTCCGC TTGCCCCCTT TTTCGGGGCA ATTTGGCCCA 3111
GGCCCTTTCC CTGCCCCGCC TAACTGCCTA AAATGGCCGC CCTGCCGTGT AGGCATTCAT 3171
CCAGCTAGAG GAATTC                                                 3187
```

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3187 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2611..3012

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

```
AGATCTGGAC CGGGGTGCTG GCCTGGGCCA CGCCGGCGAG GGCCAGCGCG GAGCAACCGA   60
GCAGCAGGGC GAGAGGTTTC ATCGGGATTC CTTGGCAGTC TGAATGACGT GCCAGCCTAT  120
CAGCGCGGCG CCGGTGCGGC GAGGGCGCGC CGGACCCAGT GCGTCACCTC TCGTCTGATC  180
CGCCTCCCTC GACGGGCGTC GCTGACAAAA AAATTCAAAC AGAAATTAAC ATTTATGTCA  240
TTTACACCAA ACCGCATTTG GTTGCAGAAT GCTCAAACGT GTGTTTGAAC AGAGCAAGCA  300
ACACGTAAAC AGGGATGACA TGCAGTACCC GTAAGAAGGG CCGATTGGCC CACAACAACA  360
CTGTTCTGCC GAACTGGAGA CCGATGATGA ATATGGACGT GATCAAGAGC TTTACCGAGC  420
AGATGCAAGG CTTCGCCGCC CCCCTCACCC GCTACAACCA GCTGCTGGCC AGCAACATCG  480
AACAGCTGAC CCGGTTGCAG CTGGCCTCCG CCAACGCCTA CGCCGAACTG GGCCTCAACC  540
AGTTGCAGGC CGTGAGCAAG GTGCAGGACA CCCAGAGCCT GGCGGCCCTG GGCACAGTGC  600
AACTGGAGAC CGCCAGCCAG CTCTCCCGCC AGATGCTGGA TGACATCCAG AAGCTGAGCG  660
```

```
CCCTCGGCCA GCAGTTCAAG GAAGAGCTGG ATGTCCTGAC CGCAGACGGC ATCAAGAAAA   720
GCACGGGCAA GGCCTGATAA CCCCTGGCTG CCCGTTCGGG CAGCCACATC TCCCCATGAC   780
TCGACGCTAC GGGCTAGTTC CCGCCTCGGG TGTGGGTGAA GGAGAGCACA TGAGCCAACC   840
ATCTTATGGC CCGCTGTTCG AGGCCCTGGC CCACTACAAT GACAAGCTGC TGGCCATGSC   900
CAAGGCCCAG ACAGAGCGCA CCGCCCAGGC GCTGCTGCAG ACCAATCTGG ACGATCTGGG   960
CCAGGTGCTG GAGCAGGGCA GCCAGCAACC CTGGCAGCTG ATCCAGGCCC AGATGAACTG  1020
GTGGCAGGAT CAGCTCAAGC TGATGCAGCA CACCCTGCTC AAAAGCGCAG GCCAGCCGAG  1080
CGAGCCGGTG ATCACCCCGG AGCGCAGCGA TCGCCGCTTC AAGGCCGAGG CCTGGAGCGA  1140
ACAACCCATC TATGACTACC TCAAGCAGTC CTACCTGCTC ACCGCCAGGC ACCTGCTGGC  1200
CTCGGTGGAT GCCCTGGAGG GCGTCCCCCA GAAGAGCCGG GAGCGGCTGC GTTTCTTCAC  1260
CCGCCAGTAC GTCAACGCCA TGGCCCCCAG CAACTTCCTG GCCACCAACC CCGAGCTGCT  1320
CAAGCTGACC CTGGAGTCCG ACGGCCAGAA CCTGGTGCGC GGACTGGCCC TCTTGGCCGA  1380
GGATCTGGAG CGCAGCGCCG ATCAGCTCAA CATCCGCCTG ACCGACGAAT CCGCCTTCGA  1440
GCTCGGGCGG GATCTGGCCC TGACCCCGGG CCGGGTGGTG CAGCGCACCG AGCTCTATGA  1500
GCTCATTCAG TACAGCCCGA CTACCGAGAC GGTGGGCAAG ACACCTGTGC TGATAGTGCC  1560
GCCCTTCATC AACAAGTACT ACATCATGGA CATGCGGCCC CAGAACTCCC TGGTCGCCTG  1620
GCTGGTCGCC CAGGGCCAGA CGGTATTCAT GATCTCCTGG CGCAACCCGG GCGTGGCCCA  1680
GGCCCAAATC GATCTCGACG ACTACGTGGT GGATGGCGTC ATCGCCGCCC TGGACGGCGT  1740
GGAGGCGGCC ACCGGCGAGC GGGAGGTGCA CGGCATCGGC TACTGCATCG GCGGCACCGC  1800
CCTGTCGCTC GCCATGGGCT GGCTGGCGGC GCGGCGCCAG AAGCAGCGGG TGCGCACCGC  1860
CACCCTGTTC ACTACCCTGC TGGACTTCTC CCAGCCCGGG GAGCTTGGCA TCTTCATCCA  1920
CGAGCCCATC ATAGCGGCGC TCGAGGCGCA AAATGAGGCC AAGGGCATCA TGGACGGGCG  1980
CCAGCTGGCG GTCTCCTTCA GCCTGCTGCG GGAGAACAGC CTCTACTGGA ACTACTACAT  2040
CGACAGCTAC CTCAAGGGTC AGAGCCCGGT GGCCTTCGAT CTGCTGCACT GGAACAGCGA  2100
CAGCACCAAT GTGGCGGGCA AGACCCACAA CAGCCTGCTG CGCCGTCTCT ACCTGGAGAA  2160
CCAGCTGGTG AAGGGGGAGC TCAAGATCCG CAACACCCGC ATCGATCTCG GCAAGGTGAA  2220
GACCCCTGTG CTGCTGGTGT CGGCGGTGGA CGATCACATC GCCCTCTGGC AGGGCACCTG  2280
GCAGGGCATG AAGCTGTTTG GCGGGGAGCA GCGCTTCCTC CTGGCGGAGT CCGGCCACAT  2340
CGCCGGCATC ATCAACCCGC CGGCCGCCAA CAAGTACGGC TTCTGGCACA ACGGGGCCGA  2400
GGCCGAGAGC CCGGAGAGCT GGCTGGCAGG GGCGACGCAC CAGGGCGGCT CCTGGTGGCC  2460
CGAGATGATG GGCTTTATCC AGAACCGTGA CGAAGGGTCA GAGCCCGTCC CCGCGCGGGT  2520
CCCGGAGGAA GGGCTGGCCC CCGCCCCCGG CCACTATGTC AAGGTGCGGC TCAACCCCGT  2580
GTTTGCCTGC CCAACAGAGG AGGACGCCGC ATG AGC GCA CAA TCC CTG GAA GTA    2634
                                Met Ser Ala Gln Ser Leu Glu Val
                                  1               5
GGC CAG AAG GCC CGT CTC AGC AAG CGG TTC GGG GCG GCG GAG GTA GCC     2682
Gly Gln Lys Ala Arg Leu Ser Lys Arg Phe Gly Ala Ala Glu Val Ala
     10                  15                  20
GCC TTC GCC GCG CTC TCG GAG GAC TTC AAC CCC CTG CAC CTG GAC CCG     2730
Ala Phe Ala Ala Leu Ser Glu Asp Phe Asn Pro Leu His Leu Asp Pro
 25                  30                  35                  40
GCC TTC GCC GCC ACC ACG GCG TTC GAG CGG CCC ATA GTC CAC GGC ATG     2778
Ala Phe Ala Ala Thr Thr Ala Phe Glu Arg Pro Ile Val His Gly Met
                 45                  50                  55
CTG CTC GCC AGC CTC TTC TCC GGG CTG CTG GGC CAG CAG TTG CCG GGC     2826
Leu Leu Ala Ser Leu Phe Ser Gly Leu Leu Gly Gln Gln Leu Pro Gly
             60                  65                  70
AAG GGG AGC ATC TAT CTG GGT CAA AGC CTC AGC TTC AAG CTG CCG GTC     2874
Lys Gly Ser Ile Tyr Leu Gly Gln Ser Leu Ser Phe Lys Leu Pro Val
         75                  80                  85
TTT GTC GGG GAC GAG GTG ACG GCC GAG GTG GAG GTG ACC GCC CTT CGC     2922
Phe Val Gly Asp Glu Val Thr Ala Glu Val Glu Val Thr Ala Leu Arg
 90                  95                  100
GAG GAC AAG CCC ATC GCC ACC CTG ACC ACC CGC ATC TTC ACC CAA GGC     2970
Glu Asp Lys Pro Ile Ala Thr Leu Thr Thr Arg Ile Phe Thr Gln Gly
105                 110                 115                 120
GGC GCC CTC GCC GTG ACG GGG GAA GCC GTG GTC AAG CTG CCT              3012
Gly Ala Leu Ala Val Thr Gly Glu Ala Val Val Lys Leu Pro
                125                 130
TAAGCACCGG CGGCACGCAG GCACAATCAG CCCGGCCCCT GCCGGGCTGA TTGTTCTCCC  3072
CCGCTCCGCT TGCCCCCTTT TTCGGGGCAA TTTGGCCCAG GCCCTTTCCC TGCCCCGCCT  3132
AACTGCCTAA AATGGCCGCC CTGCCGTGTA GGCATTCATC CAGCTAGAGG AATTC        3187
```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

AGTTCCCGCC TCGGGTGTGG GTGAA

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGCATATGCG CTCATGCGGC GTCCT 25

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GCCATATGAG CGCACAATCC CTGGAAGTAG 30

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CTGGGATCCG CCGGTGCTTA AGGCAGCTTG 30

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

```
Ser Ala Gln Ser Leu Glu Val Gly Gln Lys Ala Arg Leu Ser Lys Arg
 1               5                  10                  15
Phe Gly Ala Ala
            20
```

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

```
Met Ser Ala Gln Ser Leu Glu Val Gly Gln Lys Ala Arg Leu Ser Lys
 1               5                  10                  15
Arg Phe Gly Ala Ala
            20
```

Claims

1. A polyester synthase gene coding for a polypeptide containing the amino acid sequence of SEQ ID NO:2 or a sequence where in said amino acid sequence, one or more amino acids are deleted, replaced or added, said polypeptide bringing about polyester synthase activity.

2. A polyester synthase gene comprising the nucleotide sequence of SEQ ID NO:1.

3. A gene expression cassette comprising the polyester synthase gene of claims 1 or 2 and either of open reading frames located upstream and downstream of said gene.

4. The gene expression cassette according to claim 3, wherein the open reading frame located upstream of the polyester synthase gene comprises DNA coding for the amino acid sequence of SEQ ID NO:4.

5. The gene expression cassette according to claim 3, wherein the open reading frame located upstream of the polyester synthase gene comprises the nucleotide sequence of SEQ ID NO:3.

6. The gene expression cassette according to claim 3, wherein the open reading frame located downstream of the polyester synthase gene comprises DNA coding for a polypeptide containing the amino acid sequence of SEQ ID NO:6 or a sequence where in said amino acid sequence, one or more amino acids are deleted, replaced or added, said polypeptide bringing about enoyl-CoA hydratase activity.

7. The gene expression cassette according to claim 3, wherein the open reading frame located downstream of the

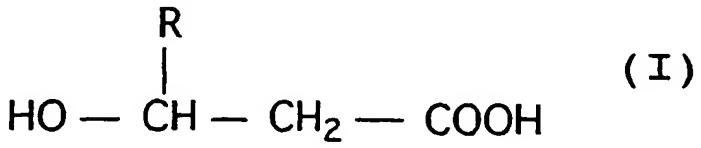
polyester synthase gene comprises the nucleotide sequence of SEQ ID NO:5.

8. A recombinant vector comprising the polyester synthase gene of claim 1 or 2 or the gene expression cassette of any one of claims 3 to 7.

9. A transformant transformed with the recombinant vector of claim 8.

10. A process for producing polyester, wherein the transformant of claim 9 is cultured in a medium and polyester is recovered from the resulting culture.

11. The process for producing polyester according to claim 10, wherein the polyester is a copolymer of 3-hydroxyalkanoic acid represented by formula I:



(I)

wherein R represents a hydrogen atom or a C1 to C4 alkyl group.

12. The process for producing polyester according to claim 10, wherein the polyester is a poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) random copolymer.

FIG. 1A

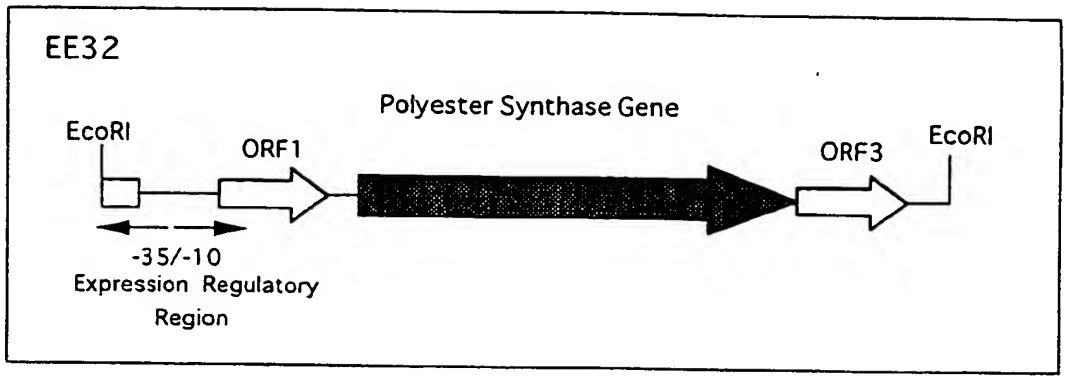


FIG. 1B

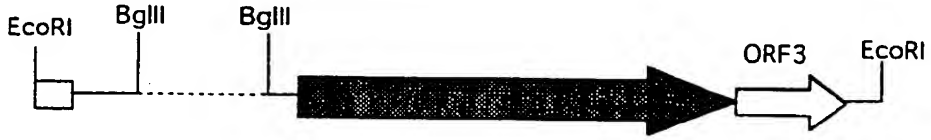


FIG. 1C

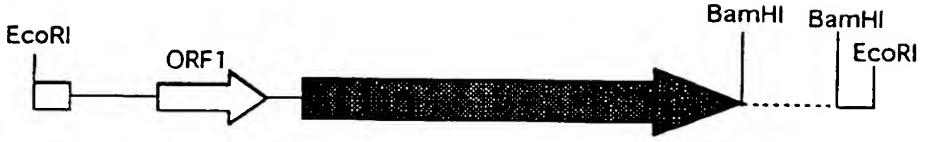
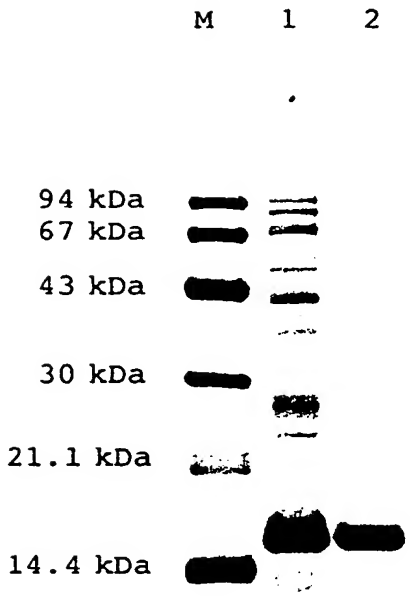


FIG. 1D

FIG.2



Lane M: molecular-weight marker

Lane 1: soluble-protein fraction from NB3

Lane 2: active fraction eluted from the anion exchange column

(19) Europäisches Patentamt
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Office européen des brevets

(11) EP 0 824 148 A3

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(83) **Declaration under Rule 28(4) EPC (expert solution)**

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(54) Polyester synthase gene and process for producing polyester

(57) The present invention relates to a polyester synthase gene coding for a polypeptide containing the amino acid sequence of SEQ ID NO:2 or a sequence where in said amino acid sequence, one or more amino acids are deleted, replaced or added, said polypeptide bringing about polyester synthase activity; a gene expression cassette comprising the polyester synthase gene and either of open reading frames located upstream and downstream of said gene; a recombinant vector comprising the gene expression cassette; a transformant transformed with the recombinant vector; and a process for producing polyester by culturing the transformant in a medium and recovering polyester from the resulting culture.

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EUROPEAN SEARCH REPORT

Application Number
EP 97 11 3932

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A	WO 93 02187 A (UNIV MICHIGAN) 4 February 1993 (1993-02-04) * claims * ---	1	C12N15/74 //(C12N1/21, C12R1:05)
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	25 August 1999	Delanghe, L

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